REMARKS

Claims 1-4 are pending and under examination. Applicants have amended claims 3 and 4. Claim 3 now recites "a therapeutic drug" while claim 4 no longer recites "various organ damages accompanied by diabetes mellitus." These amendments do not introduce new matter.

Rejection Under 35 U.S.C. § 112

The Office rejects claims 3 and 4 under 35 U.S.C. § 112, first paragraph, as allegedly not enabled. Office Action, page 2. Regarding claim 3, the Office asserts that the specification does not enable prevention of "diseases with insufficient development and regeneration of blood vessels, and various diseases caused by ischemia." *Id.* Solely to facilitate prosecution and without acquiescing to the rejection, Applicants have amended claim 3 to remove this language. As this amendment renders the Office's rejection of claim 3 moot, Applicants request that the Office withdraw this rejection.

Regarding claim 4, the Office questions whether claim 4 improperly broadens the scope of claim 3 if the concept of "organ damages accompanied by diabetes mellitus" does not fall within "diseases with insufficient development and regeneration of blood vessels, and various diseases caused by ischemia" recited in claim 3. *Id.* According to the Office, organ damage in diabetes "is not treatable by increasing blood vessel[s] since the etiology is not lacking of blood vessel[s]." *Id.* Based on these alleged premises, the Office concludes that there is "insufficient description as well as enablement as to how such damage is treatable through angiogenesis." *Id.* at 3.

Solely to facilitate prosecution and without acquiescing to the rejection, Applicants have amended claim 4 to remove the recitation of "various organ damages

accompanied by diabetes mellitus." As this amendment renders the Office's rejection of claim 4 moot, Applicants request that the Office withdraw this rejection.

Rejection Under 35 U.S.C. § 103

The Office rejects claims 1-4 under 35 U.S.C. § 103(a) as allegedly obvious over U.S. Patent 5,656,642 ("Fujioka") in view of Orito, K. et al., "Mechanisms of Action of OPC-28326, a Selective Hindlimb Vasodilator," *J. Pharmacol. Exper. Ther.* 291:604-11 (1999) ("Orito"); Da Silva-Azevedo, L. et al., "Vascular Endothelial Growth Factor Is Expressed in Endothelial Cells Isolated from Skeletal Muscles of Nitric Oxide Synthase Knockout Mice During Prazosin-Induced Angiogenesis," *Biochem. Biophys. Res. Comm.* 297:1270-6 (2002) ("Silva-Azevedo"); and Zhou, A.L. et al., "Internal Division of Capillaries in Rat Skeletal Muscle in Response to Chronic Vasodilator Treatment with α_1-Antagonist Prazosin," *Cell Tissue Res.* 293:293-303 (1998) ("Zhou") supplemented with Sumi, M. et al., "OPC-28326, a Selective Femoral Arterial Vasodilator, Augments Ischemia Induced Angiogenesis," *Biomed. Pharmacother.* 61:209-15 (2007) ("Sumi").

According to the Office, Fujioka teaches the claimed compound as useful for treating disorders that require vasodilating. Office Action, page 3. Acknowledging that Fujioka does not teach its compound for enhancing angiogenesis, the Office contends that "[v]asodilators such as prazosin not only caused vasodilation but also simultaneously affect the tissue to induce angiogenesis," citing to Silva-Azevedo and Zhou for alleged support. *Id.* Citing to Orito, the Office also suggests that Fujioka's compound and other vasodilators such as prazosin function analogously on the blood vessel. *Id.* To support the general suggestion, the Office turns to a document that is not prior art to the instant application, namely, the Sumi reference. The Office relies on

Sumi to allegedly demonstrate that the compound of Fujioka innately functions simultaneously as a vasodilator and augments angiogenesis. *Id.* at 4.

Based on these alleged teachings, the Office concludes that it would have been obvious "to employ the Fujioka et al. compounds for enhancing angiogenesis because it is the innate nature of such compounds to simultaneously [have] the affect of inducing angiogenesis." *Id.* Applicants traverse for at least the following reasons.

First, the Office's use of Sumi to provide "factual support for the well known innate nature of the Fujioka compounds" is improper. The analysis of whether an invention would have been obvious under § 103(a) is retrospective. Indeed, the MPEP instructs that "Office personnel should evaluate the prior art from the standpoint of the hypothetical person having ordinary skill in the art at the time the claimed invention was made." MPEP § 2144.08 (II)(A)(3) (citing *Ryko Mfg. Co. v. Nu-Star Inc.*, 950 F.2d 714, 718, 21 USPQ2d 1053, 1057 (Fed. Cir. 1991)). As the Office admits, Sumi was published after the filing date of the instant application and therefore does not represent what was known in the art "at the time the claimed invention was made." *See* Office Action, page 4.

Second, the Office appears to invoke the concept of inherency as a foundation for its rationale as to why claims 1-4 would have been obvious. As noted above, the Office cited to Sumi to allegedly demonstrate an "innate" feature of Fujioka's compounds and further states that "it is the innate nature of [Fujioka's] compounds to simultaneously [have] the effect of inducing angiogenesis and a compound cannot be separated from its innate nature." *Id.* Without knowing exactly what the Office intends by the use of this word, it is important to note that an allegedly inherent feature of a compound, however, cannot be used as a foundation for obviousness. As the MPEP

instructs, "[o]bviousness cannot be predicated on what is not known at the time an invention is made, even if the inherency of a certain feature is later established." MPEP § 2141.02(V) (citing *In re Rijckaert*, 9 F.2d 1531, 28 USPQ2d 1955 (Fed. Cir. 1993)). Neither Fujioka nor Orito teach or suggest that their compounds are useful for promoting angiogenesis, which is why the Office contends that Fujioka's compound and other vasodilators in general "such as prazosin function analogously on the blood vessel" and that "[v]asodilators such as prazosin not only cause vasodilation but also simultaneously affect the tissue to induce angiogenesis." Office Action, page 3. Applicants disagree with these premises.

Arguendo, even if a compound can cause vasodilation, it does not necessarily follow that the compound will also cause angiogenesis. Prazosin is an α1-adrenergic blocker. *See* Silva-Azevedo at page 1273, right column. Terazosin and Doxazosin are also α1-adrenergic blockers, having the same quinazoline structure as prazosin. Like prazosin, terazosin and doxazosin demonstrate the ability to cause vasodilation, but they *inhibit* angiogenesis. *See* Pan, S.L. et al., "Identification of Apoptotic and Antiangiogenic Activities of Terazosin in Human Prostate Cancer and Endothelial Cells" *J. Urol.* 169:724-29 (2003) and Keledjian, K. et al., "Anoikis Induction by Quinazoline Based α1-Adrenoceptor Antagonists in Prostate Cancer Cells: Antagonistic Effect of Bcl-2" *J. Urol.* 169:1150-56 (2003). Thus, the mere teaching that a α1-adrenergic blocker can cause vasodilation does not necessarily mean that it will inherently or "innately" cause angiogenesis. Applicants respectfully remind the Office that when "relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." (emphasis in

original). MPEP § 2121(IV) (citing *Ex parte Levy,* 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990)). The fact that a certain result or characteristic may occur in the prior art is not equivalent to "necessarily" and therefore is insufficient to establish the inherency of that result or characteristic. *See id.*

In sum, the failure of Fujioka and Orito to teach or suggest that their compounds are useful for promoting angiogenesis is not remedied by the alleged teachings in Silvia-Azevedo and Zhou on the activity of prazosin, as compounds that are α1-adrenergic blockers can have different effects on blood circulation and the vasculature. Sumi's teachings do not reflect what was known in the art as of the filing date of the instant application and therefore are not applicable to this rejection under § 103(a). Because the combination of Fujioka, Orito, Silva-Azevedo, Zhou, and Sumi would not have rendered claims 1-4 obvious, Applicants request that the Office withdraw this rejection.

Rejection for Obviousness-Type Double Patenting

The Office rejects claims 1-4 under the judicially created doctrine of obviousness-type double patenting in light of claims 1-8 of Fujioka in view of Orito, Silva-Azevedo, and Zhou supplemented with Sumi. Office Action, page 4. The Office uses the same rationale as discussed above for the § 103 rejection to support this double patenting rejection, noting that Fujioka and the instant application have the same Assignee. *Id.* Applicants disagree.

The Office bases this rejection on the same reasoning as used for the rejection under § 103(a) above. For at least the reasons articulated above, Applicants contend that claims 1-8 of Fujioka would not have rendered pending claims 1-4 obvious. Applicants therefore request that the Office withdraw this rejection.

Conclusions

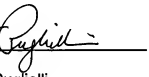
In view of the foregoing amendments and remarks, Applicants respectfully request reconsideration and reexamination of this application and the timely allowance of claims 1-4.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: June 4, 2010

By: 

Maryann T. Puglielli
Reg. No. 52,138
(202) 408-6054

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IDENTIFICATION OF APOPTOTIC AND ANTIANGIOGENIC ACTIVITIES OF TERAZOSIN IN HUMAN PROSTATE CANCER AND ENDOTHELIAL CELLS

SHIOW-LIN PAN, JIH-HWA GUH, YING-WEN HUANG, JI-WANG CHERN, JUI-YI CHOU AND CHE-MING TENG

From the Pharmacological Institutes, School of Pharmacy and Department of Urology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

ABSTRACT

Purpose: It has been suggested that terazosin has an inhibitory effect on prostate tumor growth. We determined if terazosin action contributes to direct suppression of the angiogenic effect.

Materials and Methods: PC-3 cells and primary cultures of human benign prostatic cells were used in this study. The cytotoxic effect was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and lactate dehydrogenase release reaction. The in vivo angiogenic effect was determined in nude mice models, followed by histological examination and quantification by the hemoglobin detection assay. In vitro determination of cell migration, proliferation and tube formation was performed in cultured human umbilical vein endothelial cells.

Results: Terazosin induced cytotoxicity in PC-3 and human benign prostatic cells with an IC50 of more than 100 μM. The positive terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling and lactate dehydrogenase release reaction was associated with terazosin induced cytotoxicity, indicating apoptotic and necrotic cell death. Furthermore, cytotoxicity due to terazosin action was not a common characteristic of a quinazoline based structure. Terazosin significantly inhibited vascular endothelial growth factor induced angiogenesis in nude mice with an IC50 of 7.9 μM., showing that it had a more potent anti-angiogenic than cytotoxic effect. Terazosin also effectively inhibited vascular endothelial growth factor induced proliferation and tube formation in cultured human umbilical vein endothelial cells (IC50 9.9 and 6.8 μM., respectively).

Conclusions: Together our data suggest that terazosin shows direct anti-angiogenic activity through the inhibition of proliferation and tube formation in endothelial cells. This action may partly explain the in vivo antitumor potential of terazosin.

KEY WORDS: prostate, prostatic neoplasms, endothelium, apoptosis

As members of the superfamily of G protein coupled adrenergic receptors, α1-adrenoceptors modulate diverse intracellular processes, such as activation of smooth muscle contraction, proliferation and migration of vascular smooth muscle cells, and modulation of cytoskeletal proteins in prostatic stromal cells.[1–3] The α1-adrenoceptor antagonists are administered in patients with cardiovascular diseases. In addition, they are also used for treating bladder outlet obstruction caused by benign prostatic enlargement or benign prostatic hyperplasia (BPH). They can provide symptomatic relief in patients with lower urinary tract symptoms.[4]

Terazosin, a quinazoline based α1-adrenoceptor antagonist, is one of the highly selective, long acting α1-adrenoceptor antagonists approved for symptomatic benign prostatic enlargement or BPH. Recent studies show the ability of terazosin to suppress prostate tumor growth in vivo and its therapeutic significance for androgen independent human prostate cancer.[5,6] It has been suggested that terazosin causes a significant loss of cell viability through the induction of apoptosis in PC-3 cells, which would explain its in vivo antitumor effect.[5,6] In addition, doxazosin (a quinazoline derivative) but not tamsulosin (a sulfonamide based structure) mimics terazosin induced effects, revealing the specific character of quinazoline based structures. In addition to the direct cytotoxic effect in cancer cells, the inhibition of angiogenesis has been suggested for treating solid tumors. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are regularly isolated from various cancer cell types.[7,8] Increased expression of VEGF mRNA and protein have been detected in various human tumors.[9,10] This evidence suggests that solid tumor progression depends on angiogenesis.

A study of prostate tumor vascularity in patients with BPH who have been on terazosin showed a significant decrease in prostate tissue microvascular density.[11] However, to our knowledge the direct or indirect mechanism of this terazosin action has not been identified. In the current study we investigated terazosin induced antitumor activities, such as apoptotic and anti-angiogenic effects. Furthermore, we synthesized several quinazoline based α1-adrenoceptor antagonists and examined their anticancer activity in PC-3 cells to determine the structure.

MATERIALS AND METHODS

Materials. We used RPMI-1640, M199 medium, other cell culture reagents, terazosin, a hemoglobin assay kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), type I collagen and [^{3}H]thymidine. We also used a series of

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quinazoline derivatives designed and synthesized as α1-adrenoceptor antagonists. FH-71 (ethyl 4-(3-(4-(2-methoxyphenyl) piperazinyl) aminoquinazolin-2-carboxylate), EW-65 (4-(3-(4-(2-methoxyphenyl) piperazinyl) propyl) aminoquinazolin-2- carboxamide) and EW-154 (2-(4-(4-(2-methoxyphenyl) piperazinyl)butylamino-4- cyclohexylaminoquinazolin) were assessed in the current study (fig. 1).

Cell cultures. Human prostate adenocarcinoma PC-3 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (volume per volume) and penicillin (100 units $ml.^{-1}$)/streptomycin (100 $\mu g. ml.^{-1}$). Cultures were maintained in a humidified incubator at 37C in 5% CO_2/air. Human prostatic tissue explants were obtained and smooth muscle cells were cultured, as previously described.[12] Human umbilical vein endothelial cells obtained by treating human umbilical cord veins with collagenase were cultured in 75 $cm.^2$ plastic flasks in M199 containing 20% fetal bovine serum and 15 $\mu g. ml.^{-1}$ endothelial cell growth supplements. The cytotoxic reaction was assessed using the MTT assay method, as previously described.[12]

Assessment of lactate dehydrogenase (LDH) release. Necrotic cell death was measured by the release of LDH into the culture medium, indicating the loss of membrane integrity and cell necrosis. LDH activity was measured using a commercial cytotoxicity assay kit (Promega, Madison, Wisconsin), in which released LDH in culture supernatants is measured with a coupled enzymatic assay, resulting in conversion of a tetrazolium salt into a red formazan product. The necrotic percent was expressed using the formula, (sample value/maximal release) × 100%. Maximal release was obtained after treating control cells with 0.5% Triton X-100 for 10 minutes at room temperature.

In vivo angiogenesis model. Matrigel (Becton Dickinson Lab., Bedford, Massachusetts) (0.5 ml.) containing vehicle, VEGF or VEGF plus terazosin was subcutaneously injected into nude mice to determine the anti-angiogenic activity of terazosin. After 6 days of incubation the animals were sacrificed by intraperitoneal administration of pentobarbital. The Matrigel plug was clipped for histological examination and angiogenesis determination using a hemoglobin assay kit. For histological examination the plug was placed into 4% paraformaldehyde and embedded in paraffin. Embedded tissues were sectioned at 6 μm., stained with hematoxylin and eosin, and analyzed by microscopy.

Cell migration assay. The migration assay was measured with a modified Boyden chamber assay in Transwell (Costar, Cambridge, Massachusetts) culture chambers, as previously described.[13] VEGF was diluted (10 $ng. ml.^{-1}$) in M199/0.1% bovine serum albumin and loaded into the lower wells of the chamber in duplicate. The wells were covered with a polycarbonate filter with 8 μm. pores (Costar) coated with 500 $\mu g. ml.^{-1}$ Matrigel. Cultured human umbilical vein endothelial cells (1×10^5 cells in 100 μl. M199/0.1% bovine serum albumin) were added into the upper wells of the chamber in the absence or presence of terazosin. The chambers were incubated for 24 hours at 37C in an atmosphere of 95% air and 5% CO_2. At the end of incubation the cells were fixed and stained with hematoxylin. Nonmigrated cells on the top of the filters were wiped off with cotton swabs, the filters were mounted and migrated cells attached to the bottom of filters were counted in 6 random high power fields (magnification 100×). Cell migration was calculated as the difference in the number of migrated cells in the terazosin treated and control groups.

[^{3}H]thymidine incorporation assay. Cells were incubated with or without indicated reagents for 24 hours and then harvested for the detection of DNA synthesis. Before harvest cells were incubated with [^{3}H]thymidine (1 $\mu Ci. ml.^{-1}$) for 4 hours. They were then processed and harvested with FilterMate Packard Instrument Co., Meriden, Connecticut) and incorporated radioactivity was determined.

Tube formation assay. Cultured human umbilical vein endothelial cells were cultured into slide chambers pre-coated with Matrigel. Cells were treated with vehicle, VEGF or VEGF plus terazosin for 24 hours and tube formation was observed by microscopy. To quantify tube formation 3 random areas were imaged and the total tube length per area was quantified using Image-Pro Plus, version 3.0 (Media Cybernetics, Inc., Baltimore, Maryland) image analysis software.

Statistical analysis. Data are presented as the mean ± SEM for the indicated number of experiments. Statistical analysis of data were performed with 1-way ANOVA, followed by the t test with $p < 0.05$ considered significant.

Terazosin

FH-71

EW-65

EW-154

FIG. 1. Chemical structure of terazosin, FH-71, EW-65 and EW-154.

RESULTS

Effect of terazosin on the cytotoxic effect. The effect of terazosin on the cytotoxic effect in the androgen independent human prostate cancer cell line PC-3 was examined. Data showed a concentration dependent loss of cell viability with an IC50 of more than 100 μm. (fig. 2, *A*). In addition, prazosin (30 μM.) also showed little cytotoxicity in PC-3 cells in 4 preparations compared with controls (94.1% ± 1.8% cell survival). Moreover, the terazosin induced cytotoxic effect is not a specific action in cancer cells since similar cytotoxicity was detected in a primary culture of human prostate smooth muscle cells (fig. 2, *B*).

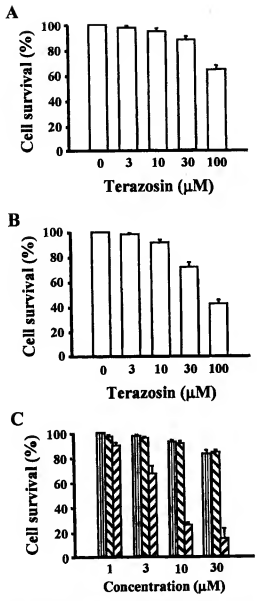

FIG. 2. Effect of terazosin, FH-71, EW-65 and EW-154 on cytotoxicity of PC-3 (*A* and *C*) and human benign prostatic cells (*B*) treated with or without terazosin (*A* and *B*), FH-71 (▨), EW-65 (▩) and EW-154 (▩) (*C*) for 24 hours, as assessed by MTT assay. Results are expressed as percent cell survival in control. Data are shown as mean ± SEM of 4 determinations.

To determine the cytotoxic effect mode of action several identification techniques were used in the current study. Apoptotic cells were detected in situ using terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling. Results showed a positive reaction after a 12-hour treatment of PC-3 cells with terazosin (100 μM.) (data not shown). However, in 3 preparations exposure of PC-3 cells to this concentration of terazosin also showed a modest but significantly elevated LDH release reaction versus controls (19.7% ± 1.8% versus 9.0% ± 0.9%, $p < 0.001$), indicating an apoptotic and necrotic reaction at the high concentration of terazosin.

In the other experiment to determine if terazosin induced cytotoxicity was a specific character of the quinazoline based structure we synthesized several quinazoline based α1-adrenoceptor antagonists, including FH-71, EW-65 and EW-154, and examined their cytotoxic effects in PC-3 cells. Only EW-154 showed profound cytotoxicity, negating the quinazoline based character (fig. 2, *C*).

Effect of terazosin on VEGF induced angiogenesis in an in vivo model. Nude mice models were used in this study to examine the direct in vivo effect of terazosin on regulation of the angiogenic effect. The subcutaneously inserted Matrigel plug showed a marked angiogenic effect after 6 days of VEGF incubation (fig. 3). However, terazosin significantly diminished angiogenesis due to VEGF action. The in vivo angiogenic effect was also quantitatively analyzed using the hemoglobin detection assay. Data showed that terazosin inhibited the VEGF induced angiogenic effect in a concentration dependent manner with an IC50 of 7.9 μM. (fig. 4).

Effect of terazosin on cell migration in cultured human umbilical vein endothelial cells. The Transwell culture chamber assay was used to determine the influence of terazosin on migration in cultured human umbilical vein endothelial cells. VEGF induced a profound increase of migrated cells, while 3 and 10 μM. terazosin had no statistically significant influence on the VEGF induced effect (fig. 5, A to C). However, it induced marked inhibition of VEGF action at the higher concentration of 30 μM. (fig. 5, *D*).

Effect of terazosin on the regulation of cultured human umbilical vein endothelial cell proliferation. The effect of terazosin on the regulation of endothelial cell growth was examined in primary cultures of cultured human umbilical vein endothelial cells. Exposure of cells to VEGF (10 ng. ml^{-1}) for 24 hours induced a profound increase in DNA synthesis on the [^{3}H]thymidine incorporation assay. The VEGF induced effect was inhibited by terazosin in a concentration dependent manner with an IC50 of 9.9 μM. (fig. 6). Furthermore, even at the high concentration of 30 μM. terazosin had only a negligible influence on basal cell survival on the MTT assay and LDH release reaction (data not shown). This observation suggests that terazosin mediated inhibition of endothelial cell growth was not achieved through the cytotoxic effect.

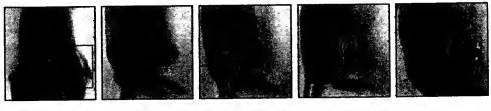

FIG. 3. Effect of terazosin on VEGF induced angiogenesis in nude mice models. Matrigel plugs without (*Basal*) or with VEGF in absence (*Control*) or presence of terazosin were subcutaneously injected. After 6-day incubation plugs were clipped off for photo observation. Experiments were done in 4 mice per group.

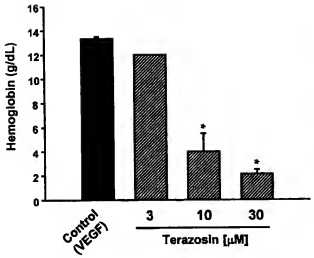

FIG. 4. Quantitative determination of terazosin induced anti-angiogenic effect in nude mice models. Animals were treated and Matrigel plugs were clipped off for assessment of angiogenic effect using hemoglobin assay kit. Data are expressed as mean ± SEM of 4 determinations. Asterisk indicates $p < 0.001$ versus VEGF control.

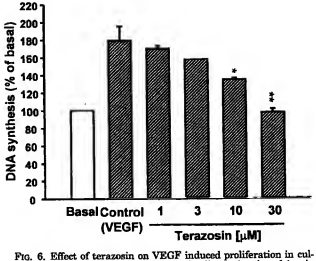

FIG. 6. Effect of terazosin on VEGF induced proliferation in cultured human umbilical vein endothelial cells pre-incubated in absence (*Basal* and *Control*) or presence of terazosin, treated without (*Basal*) or with VEGF for 24 hours and harvested for detection of DNA synthesis using [^{3}H]thymidine incorporation assay, as described. Data are expressed as mean ± SEM of 4 determinations. Single asterisk indicates $p < 0.05$ versus VEGF control. Double asterisks indicate $p < 0.001$ versus VEGF control.

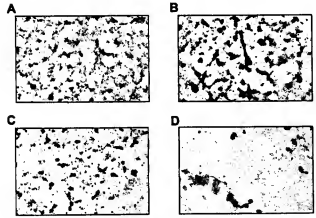

FIG. 5. Effect of terazosin on VEGF induced migration in cultured human umbilical vein endothelial cells. VEGF was loaded into lower wells of Transwell chamber in duplicate and cells were added into upper wells in absence (*A*) or presence of 3 (*B*), 10 (*C*) or 30 (*D*) μM terazosin. Chambers were incubated for 24 hours at 37C, fixed and stained as described. Migrated cells attached to bottom of filters were observed by microscopy. Reduced from ×100.

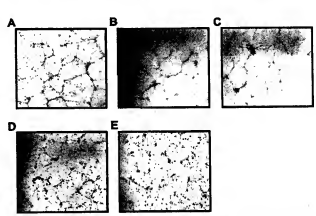

FIG. 7. Effect of terazosin on VEGF induced tube formation in cultured human umbilical vein endothelial cells cultured into slide chambers precoated with Matrigel and pre-incubated in absence (*A*) or presence of 1 (*B*), 3 (*C*), 10 (*D*) or 30 (*E*) μM. terazosin. VEGF was added to cells for 24 hours. After incubation tube formation was observed by microscopy. Reduced from ×40.

Effect of terazosin on tube formation in cultured human umbilical vein endothelial cells. Tube formation in endothelial cells is one of the most crucial events in angiogenesis. In the current series we also examined the effect of terazosin on tube formation in endothelial cells. In 3 preparations 1, 3, 10 and 30 μM. terazosin showed 2.4% ± 0.6%, 20.3% ± 4.3%, 70.7% ± 8.3% and 91.6% ± 1.9% inhibition of tube formation, respectively, with a calculated IC50 of terazosin action of 6.8 μM. (fig. 7). Again terazosin induced suppression of tube formation was not achieved via the cytotoxic effect since cells at any concentration of terazosin showed the profound formation of dark blue formazan crystals on MTT loading (fig. 7).

DISCUSSION

Several lines of evidence suggest that quinazoline derived α1-adrenoceptor antagonists induce the apoptotic reaction in human benign prostatic and prostate cancer cells.[6,14,15] Apoptosis mediated regression of prostate volume and tumor size is thought to be a molecular mechanism underlying the therapeutic potential of α1-adrenergic blockade for treating BPH and prostate cancer. However, these reports and related experiments have raised several questions and interests at our laboratory. The first question is whether the apoptotic reaction is a common characteristic of these quinazoline based α1-adrenoceptor antagonists. To resolve this question several quinazoline based α1-adrenoceptor antagonists, including FH-71, EW-65 and EW-154, were synthesized (fig. 1). Their cytotoxic effects in human prostate cancer cells were examined in the current study.

In our unpublished data the antagonistic characterization of these 3 compounds to α1-adrenoceptors was determined using radioligand binding and functional studies. However, in the current series only EW-154 showed profound cytotoxicity in PC-3 cells, indicating that quinazoline may not be the lead structure eliciting apoptosis in human prostate cancer

cells. In addition, terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling and the LDH release reaction were used to examine the cytotoxic mode of terazosin in PC-3 cells. There was a positive reaction in each method, revealing that terazosin induced cytotoxicity is not simply the apoptotic reaction. However, we need further studies of more quinazoline and nonquinazoline based compounds to draw a conclusion.

Furthermore, to investigate if the mechanism to terazosin action in PC-3 cells depends on the ability to antagonize α1-adrenoceptor we also examined the prazosin induced effect in this study. Prazosin was more potent than terazosin on α1-adrenoceptor antagonizing activity (about 10-fold). However, it showed little cytotoxic effect in PC-3 cells. Furthermore, in our previous study EW-154 was less potent than FH-71 and terazosin against human prostatic muscle contraction caused by α1-adrenoceptor activation, whereas it showed the most effective cytotoxicity in PC-3 cells.[16] Data revealed that the cytotoxic effect is not parallel with α1-adrenoceptor antagonizing activity, suggesting that the cytotoxic effects of terazosin and EW-154 are not achieved through their blocking activities on α1-adrenoceptors.

The other question is whether the in vivo antitumor effect can be simply explained by the apoptotic reaction to terazosin action. Recently evidence has shown that solid tumor progression depends on vascularity around the tumors and the inhibition of angiogenesis results in the suppression of tumor growth.[17,18] In the study of Keledjian et al the administration of terazosin decreased prostate tumor vascularity in patients with BPH and cancerous areas in a benign prostate gland.[13] In that report tissue prostate specific antigen (PSA) expression in prostatic tumor foci was profoundly decreased after terazosin treatment, suggesting that tissue PSA does not have a role in the regulation of prostate tumor vascularity since evidence indicates that PSA has a potent anti-angiogenic effect in prostate tumors.[19] Furthermore, little change in VEGF expression was detected, suggesting that the terazosin induced decrease in tumor vascular density is not associated with the alteration in VEGF protein expression. However, an interesting observation in that report was that linear regression analysis showed that microvessel density significantly correlated negatively with an increased apoptotic index of tumor areas in terazosin treated prostate specimens.[13] The apoptotic index may result from the direct apoptotic effect or be due to the inhibition of tumor vascularity caused by terazosin action. In our current series we noted that terazosin directly inhibited the VEGF induced angiogenic effect in an in vivo model. In addition, terazosin showed more potent anti-angiogenic than direct apoptotic activity in human prostate cancer cells. However, these 2 assays (anti-angiogenesis and cytotoxicity) are based on different parameters and, thus, they differed in the current study. However, anti-angiogenic and direct cytotoxic effects of terazosin action were noted. Further investigation is needed to determine the most predominant mechanism of action that results in the in vivo antitumor effect.

We also examined the effect of terazosin on angiogenesis related events in human endothelial cells. Several angiogenic factors effectively induce endothelial cells to secrete proteases, which cause degradation of the vessel basement membrane and allow cells to invade the surrounding matrix. In response to the angiogenic factors, such as bFGF and VEGF, endothelial cells migrate, proliferate and finally differentiate to a new vessel.[20] The angiogenic effect is a complex process. However, we determined the effect of terazosin on 3 functions, namely migration, proliferation and tube formation, in endothelial cells. Only at the concentration of 30 μM. terazosin was there profound inhibition in VEGF induced cultured human umbilical vein endothelial cell migration (fig. 5). A similar result was also detected in the in vivo animal model. On in vivo angiogenesis assessment, although angiogenesis was significantly inhibited by 10 μM. terazosin on hemoglobin assay, profound cell migration was still observed on histological examination (fig. 4).

We examined the effect of terazosin on serum induced proliferation in rat aortic smooth muscle cells. We found that up to the concentration of 100 μM. terazosin caused little inhibition of proliferation but showed modest cytotoxicity (about 36%) in this cell type. In contrast, terazosin induced profound inhibition of cultured human umbilical vein endothelial cell proliferation with an IC50 of 9.9 μM. These data indicate that endothelial cells are more susceptible to terazosin action. Interestingly in a parallel experiment terazosin also showed concentration dependent inhibition of bFGF induced cultured human umbilical vein endothelial cell proliferation on the [^{3}H]thymidine incorporation assay (data not shown). IC50 was calculated to be 16 μM. These data indicate that terazosin induced inhibition of the angiogenic effect is not likely to be achieved via the specific blockade of VEGF signaled pathways. However, defining the common and crucial signaling pathway by which VEGF and bFGF mediate angiogenesis has become the major challenge to investigators, including our group, performing research in the field of angiogenesis as well as further investigation in the current study.

CONCLUSIONS

We suggest that terazosin may exert its antitumor activity via a direct cytotoxic effect on human prostate cancer cells and the inhibition of angiogenesis. Terazosin induced cytotoxicity is not a common characteristic of its quinazoline structure. Furthermore, terazosin shows more potent anti-angiogenic effect in endothelial cells than cytotoxicity in human prostate cancer cells.

RPMI-1640 and M199 medium and all other cell culture reagents were obtained from Gibco, Grand Island, New York. Terazosin, the hemoglobin assay kit, MTT and type I collagen were obtained from Sigma Chemical Co., St. Louis, Missouri. [^{3}H]thymidine was obtained from American Radiolabeled Chemicals, St. Louis, Missouri. The series of quinazoline derivatives was designed and synthesized as α1-adrenoceptor antagonists by Dr. Ji-Wang Chern.

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ANOIKIS INDUCTION BY QUINAZOLINE BASED α1-ADRENOCEPTOR ANTAGONISTS IN PROSTATE CANCER CELLS: ANTAGONISTIC EFFECT OF BCL-2

KASPAR KELEDJIAN AND NATASHA KYPRIANOU

From the Division of Urology, University of Maryland, Baltimore, Maryland, and the Division of Urology and Department of Molecular Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky

ABSTRACT

Purpose: The ability of the quinazoline derived α1-adrenoceptor antagonists doxazosin and terazosin to induce apoptosis in benign and malignant prostate cells has been established. In this study we investigated the effect of the 2 piperazidinyl quinazoline based α1-adrenoceptor antagonists and the methoxybenzene sulfonamide α1-antagonist tamsulosin on human prostate cancer cell adhesion.

Materials and Methods: Androgen independent PC-3 prostate cancer cells and PC-3 transfectant clones over expressing the apoptosis suppressor bcl-2 were used as an in vitro model. Cells were treated with pharmacologically relevant doses of 1 of the 3 α1-adrenoceptor antagonists and the effect on cell viability/cell adhesion on various substrates was examined. Analysis of expression of key attachment factors, such as vascular endothelial growth factor (VEGF) and hypoxia inducible factor-α, was performed.

Results: Our results indicate a significant decrease in prostate cancer cell adhesion to gelatin and collagen but not to fibronectin in prostate cancer cells treated with doxazosin or terazosin (25 μM.) compared with untreated control cultures ($p < 0.05$). In contrast, tamsulosin had no effect on prostate cancer cell adhesion. The 2 quinazolines doxazosin and terazosin but not tamsulosin had a significant inhibitory effect on prostate tumor cell invasion. In bcl-2 over expressing prostate cancer cells there was significant suppression of doxazosin induced anoikis and cell invasion compared with neocontrol transfectants ($p < 0.05$). Doxazosin resulted in transient down-regulation (2-fold decrease) of VEGF at the mRNA and protein levels, as detected by reverse transcriptase-polymerase chain reaction and Western blotting, respectively. No significant changes in the expression profile of hypoxia inducible factor-1α were observed after treatment with quinazolines. Furthermore, bcl-2 resulted in partial reversion of the doxazosin induced VEGF decrease.

Conclusions: These findings demonstrate that the quinazoline derived α1-antagonists doxazosin and terazosin but not sulfonamide based tamsulosin induce anoikis and inhibit prostate cancer cell invasion, an effect that is antagonized by bcl-2. This molecular basis of an α1-adrenoceptor independent action against prostate cancer cells by the quinazolines may have potential therapeutic significance in prostate cancer.

KEY WORDS: prostate, prostatic neoplasms, quinazolines, apoptosis, cell adhesion

Approximately 180,400 American men were diagnosed with prostate cancer last year and 31,900 died of the disease.[1] Conventional treatment approaches include surgery, androgen ablation monotherapy and radiotherapy, which result in prolonged survival and in some patients cure by eradicating prostate tumor cells.[2,3] However, tumor relapse and metastasis occur frequently and in a majority of patients prostate cancer becomes unresponsive to subsequent hormonal therapy due to the emergence of androgen resistant tumors.[4] Clinical protocols using chemotherapy or combination treatments with androgen deprivation and chemotherapy show some therapeutic promise for treating advanced disease but there has been a limited successful impact on disease-free survival.[3] Understanding the molecular pathways of prostate cancer progression has led to intensified efforts for the development of chemotherapeutic strategies for prostate cancer by targeting key regulators of apoptosis.[4]

A critical step in the metastatic process is the adherence of tumor cells to organ microvasculature and further extravasation.[5,6] Cell adhesion to extracellular matrix (ECM) is mediated by the integrin family of cell receptors,[6,7] which elicit intracellular signals at attachment, regulating cell growth, differentiation and cell survival.[7,8] Most epithelial cells and endothelial cells undergo apoptosis when they lose contact with the ECM or bind through an inappropriate integrin.[5,8] Disruption of proper integrin ligand interactions leads to the phenomenon of anoikis (the Greek word for homelessness), a term that describes apoptosis induced by the loss of attachment to the ECM.[7,9] Anoikis resistance arises from loss of apoptotic signaling via inhibition of caspase activity and over expression of bcl-2[9] or the activation of integrin signaling.[8,10] Agents that induce endothelial or epithelial cell apoptosis by antagonizing integrin binding are considered for cancer therapy because of their ability to inhibit tumor vascularization.[10]

Tumors attempt to escape the effects of hypoxia through angiogenesis stimulation and apoptosis inhibition. Recent evidence identified the role of hypoxia inducible factor in apoptosis and tumor angiogenesis.[11] Hypoxia inducible factor-1α has been associated with prostate cancer devel-

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opment, progression and metastasis, potentially via its vital contribution toward tumor vascularization.[11] Vascular endothelial growth factor (VEGF) is also a potent promoter of tumor neovascularization.[12] Tumor vasculature regresses rapidly after VEGF down-regulation, a gene that appears to be under androgenic regulation in the malignant prostate. Loss of hypoxia inducible factor-1α reduces hypoxia induced VEGF expression and impairs vascular function, resulting in hypoxic microenvironments within the tumor mass.[12]

Clinical evidence established the use of α1-adrenoceptor antagonists as standard medical therapy for benign prostatic hyperplasia[13] since these drugs safely provide long-term relief of lower urinary tract symptoms with high efficacy. This therapeutic benefit was believed to involve targeting of the periurethral tone of the prostate, thus, causing relaxation of the prostate smooth muscle component.[13] However, recent evidence indicates that the quinazoline based, α1-adrenoceptor antagonists doxazosin and terazosin induce apoptosis in benign and malignant prostate cells via α1-adrenoceptor independent action.[14–17] Interestingly the chemically distinct α1-adrenoceptor antagonist tamsulosin (a sulfonamide derivative) has no significant effect on prostate growth.[16] In addition, terazosin results in reduced human prostate tumor vascularity.[18] All 3 α1-adrenoceptor antagonists are in clinical use for benign prostatic hyperplasia, while quinazoline based α1-adrenoceptor antagonists are used for treating hypertension.[13]

Enforced bcl-2 expression severely suppresses apoptotic cell death[18] and increased bcl-2 expression correlates with the development of androgen independent prostate cancer, an apoptotic defect that renders these tumors resistant to therapy.[4] Considering the therapeutic benefit of apoptosis induction within the context of tumor neovascularization inhibition, in this study we hypothesized that quinazoline based α1-adrenoceptor antagonists induce anoikis in human prostate cancer cells and this effect is regulated by bcl-2. Our results suggest that piperazinyl quinazolines but not tamsulosin inhibit prostate tumor cell invasion via anoikis induction.

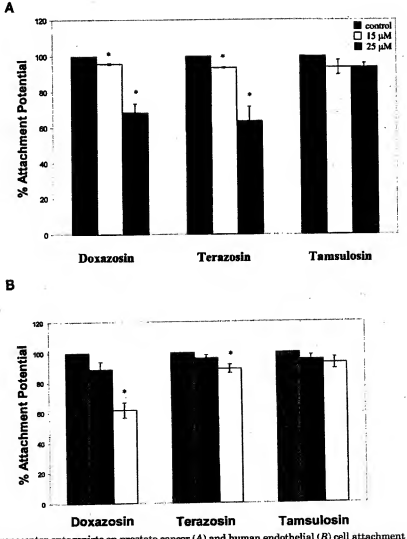

FIG. 1. Effect of α1-adrenoceptor antagonists on prostate cancer (*A*) and human endothelial (*B*) cell attachment on gelatin. PC-3 cells and HBME-1 cells were treated with 15 or 25 μM. doxazosin, terazosin or tamsulosin for 10 minutes on gelatin coated 6-well plates. Attached cells were fixed with total methanol, visualized and counted under light microscopy. Values represent mean of 3 independent assays performed in duplicate. Asterisk indicates $p < 0.05$.

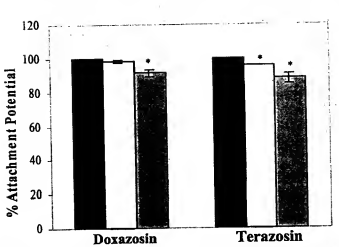

FIG. 2. Effect of quanizoline based α1-adrenoceptor antagonists on PC-3 prostate cancer cell attachment on collagen coated plates. Cells were treated with doxazosin or terazosin. Attached cells were fixed with methanol and counted under light microscopy. Asterisk indicates $p < 0.05$.

MATERIALS AND METHODS

Cell culture. Certain cell lines were used, including PC-3 human prostate cancer cells and PC-3/neomycin control transfectants. PC-3/bcl-2 cloned transfectants have been previously generated and characterized at this laboratory. PC-3/bcl-2 (clone 18) over expresses bcl-2 protein and it was routinely used for the proposed experiments. In addition, we used HBME-1 human bone marrow endothelial cells. Cells were cultured in RPMI-1640 (Gibco BRL, Gaithersburg, Maryland) medium supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) and antibiotics at 37C with 5% CO_2.

Drugs. We used 3 α1-adrenoceptor antagonists in this study, namely doxazosin mesylate, terazosin hydrochloride and tamsulosin hydrochloride.

Cell viability assay. Subconfluent cultures of PC-3 parental, PC-3/neo transfectants and PC-3/bcl-2 expressing cells were exposed to increasing concentrations of doxazosin, terazosin or tamsulosin (0.1 to 50 μM.). Cell viability was assessed after 48 hours using the trypan blue exclusion assay. Values are expressed as the mean percent of cell viability relative to untreated cultures. Each assay was performed in duplicate.

Attachment assays. Subconfluent cultures of PC-3 parental cells, PC-3/neo, PC-3/bcl-2 transfectants and HBME-1 cells were treated with doxazosin, terazosin or tamsulosin (15 and 25 μM.). After 24 hours cells were seeded in 6-well plates at 10^5 cells per well coated with gelatin (Sigma Chemical Co., St. Louis, Missouri), fibronectin (3.2 μg./cm.^{2}) or type I collagen (BD Biosciences Discovery Labware, Bedford, Massachusetts) (1 μg./cm.^{2}). After a 10-minute attachment period at 37C in 5% CO_2 attached cells were fixed with methanol and maintained at 4C for image analysis. Cells in 3 fields were counted and the average was calculated. Each assay was independently performed 3 times.

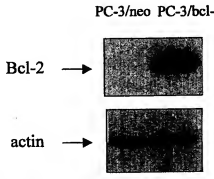

FIG. 3. Western blot analysis of bcl-2 expression in PC-3/bcl-2 prostate cancer transfectant cells. Cell lysates (30 μg.) from PC-3/neo and PC-3/bcl-2 cloned transfectants were electrophoretically analyzed and bcl-2 expression was detected using monoclonal antibody.

Preparation of nuclear extracts. Cells were treated with doxazosin (25 μM.) for 0 to 24 hours and subsequently exposed to 100 μM. $CoCl_2$. Cells were washed with tris-buffered saline and pelleted by centrifugation at 1,500 × gravity for 5 minutes. Pellets were re-suspended in Buffer A, composed of 10 mM. HEPES, pH7.9, 10 mM. KCl, 0.1 mM. ethylenediaminetetracetic acid, 0.1 mM. egtazic acid, 1 mM. dithiothreitol and 0.5 mM. phenylmethylsulfonyl fluoride. The nuclear pellet was re-suspended in buffer C (20 mM. HEPES pH 7.9, 400 mM. NaCl, 1 mM. dithiothreitol, 1 mM. ethylenediaminetetracetic acid, 1 mM. egtazic acid and 1 mM. phenylmethylsulfonyl fluoride at 4C, and centrifuged at 11,000 × gravity for 5 minutes. Protein content was evaluated using a protein kit (BioRad Laboratories, Hercules, California).

Western blotting. Cell lysates were prepared from doxazosin treated and untreated cultures of PC-3/neo and PC-3/bcl-2 cells (25 μM.) for 0 to 24 hours. Cells were harvested and lyzed with RIPA buffer composed of 150 mM. NaCl, 50 mM. tris, pH 8.0, 0.5% deoxycholic acid, 1% NP-40 and a protease inhibitor. Protein samples (30 μg.) were subjected to electrophoretic analysis through 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to Hybond P (Amersham Pharmacia Biotech, Piscataway, New Jersey) membrane. Membranes were incubated with anti-VEGF polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California). For hypoxia inducible factor-α detection nuclear extracts were subjected to electrophoresis analysis on 7.5% polyacrylamide gel, transferred to Hybond P membranes and exposed overnight to antihypoxia inducible factor-1α monoclonal antibody (Novus Biologicals, Inc., Littleton, Colorado). After incubation with the respective primary antibodies the membranes were incubated with species specific secondary antibodies. The membranes were subsequently incubated with an enhanced chemiluminescence system (Amersham Pharmacia Biotech) and autoradiographed using x-ray film. Densitometry analysis was performed using the Scion Image (Scion Corp., Frederick, Maryland) program. Bands were

normalized to α-actin expression using CP01 (Calbiochem, San Diego, California) and shown as the fold change of untreated samples.

Reverse transcriptase-polymerase chain reaction (RT-PCR). PC-3 and PC-3/bcl-2 cells were treated with doxazosin for various periods. Total RNA was prepared using the Trizol (Life Technologies, Inc., Gaithersburg, Maryland) method. The SuperScript cDNA Preamplification System (Life Technologies, Inc.) was used to generate cDNA according to manufacturer instructions. PCR was performed for 40 cycles at 94C for 1 minute, 62C for 1 minute and 72C for 2 minutes with final extension at 72C for 10 minutes using the primers 5'-TGCACCCATGGCAGAAGGAGG-3' and 5'-TCACCGCCTCGGCTTGTCACA-3', designed to detect VEGF mRNA splice variants. Glyceraldehyde-3-phosphate dehydrogenase served as a loading control. PCR products were electrophoresed on 1% (weight per volume) agarose gels and photographed under ultraviolet illumination.

Migration and invasion assays. BD Falcon Fluoroblok 24-well inserts coated with Matrigel on a light tight, 8 μ. pore size fluorescent blocking membrane (BD Biosciences Discovery Labware) were used for invasion assays. For control experiments 24-well inserts without Matrigel were used. Plates were prepared by rehydrating the Matrigel coating with PBS for 2 hours at 37C. After PBS removal 750 μl. medium containing chemoattractant (10% fetal bovine serum) was added to each well and 5×10^4 cells were added to each insert. Drugs were added to the medium (25 μM.) in the upper and lower chambers with the cells and chemoattractant solution. After 24 hours the insert plate was transferred to another 24-well plate containing 0.5 ml. per well 4 μg./ml. Calcein AM (Molecular Probes, Eugene, Oregon). Plates were read in a fluorescence plate reader (Perkin Elmer Wallac Victor, Wellesley, Massachusetts) [2] using excitation/emission wavelengths of 485/535 nm. Invasion is expressed as the mean percent cell invasion derived using the equation, mean cell invasion through Matrigel coated inserts/mean FU cell migration through uncoated control inserts in FU.

Statistical analysis. One-way ANOVA was performed to determine the statistical difference between values. Statistical analysis was performed using the Microcal Origin (MicroCal, LLC, Northampton, Massachusetts) statistical program. All data are represented as the average ± SEM with $p < 0.05$ considered statistically significant.

RESULTS

To identify the effect of α1-adrenoceptor antagonists on prostate cancer cell attachment in vitro adhesion assays were performed on 3 major ECM components, that is gelatin, collagen and fibronectin. Figure 1, *A* indicates the attachment ability of PC-3 prostate cancer cells after treatment with the 3 α1-adrenoceptor antagonists. Cell attachment on gelatin coated plates was significantly inhibited by doxazosin and terazosin, while tamsulosin had no effect on PC-3 cell adhesion ($p < 0.05$, fig. 1, *A*). The ability of α1-adrenoceptor antagonists to regulate endothelial cell adhesion was also investigated. HBME-1 human bone marrow endothelial cells were exposed to the 3 drugs and cell attachment on gelatin coated matrix was evaluated. Figure 1, *B* shows that doxazosin and terazosin treatment resulted in significant abrogation of endothelial cell attachment, similar to that of prostate tumor epithelial cells. On the other hand, tamsulosin did not affect endothelial cell adhesion (fig. 1, *B*).

On collagen coated plates doxazosin and terazosin had a modest effect on PC-3 cell adhesion that achieved statistical significance compared with untreated cells at high doses ($p < 0.05$). Figure 2 shows that doxazosin and terazosin resulted in 10% and 13% inhibition of cell attachment, respectively. In contrast, no effect on PC-3 cell attachment on fibronectin coated plates was observed for any of the drugs investigated (data not shown).

The effect of bcl-2 on inhibition of prostate cancer cell adhesion by quinazoline derived α1-adrenoceptor antagonists was subsequently determined using attachment assays. Stable cloned PC-3/neocontrol and PC-3/bcl-2 transfectants were used (fig. 3). Data revealed that bcl-2 over expression had a significant protective effect on the quinazoline induced loss of cell attachment on gelatin of PC-3 prostate cells ($p < 0.05$, fig. 4). In subsequent experiments the effect of doxazosin on prostate cell growth was comparatively evaluated in PC-3/neo and PC-3/bcl-2 cells. Figure 5 shows the dose dependent loss of viability of prostate cancer cells. A similar profile of doxazosin induced cell death was obtained for PC-3/neo and PC-3/bcl-2 cells (fig. 5).

We subsequently hypothesized that quinazoline based compounds antagonists may affect prostate cancer cell attachment by regulating the expression of 2 key angiogenesis factors controlling the migration and invasion potential of tumor epithelial and endothelial cells, namely VEGF and hypoxia inducible factor-1α. RT-PCR revealed a significant

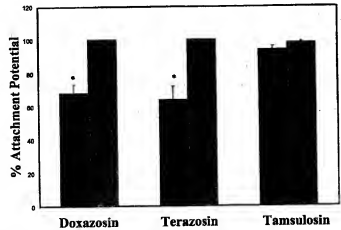

FIG. 4. Effect of bcl-2 over expression on α1-adrenoceptor antagonist induced inhibition of PC-3 and PC-3/bcl-2 prostate cancer cell attachment. Cells were treated with 25 μM. doxazosin, terazosin and tamsulosin. Data are expressed as percent cell attachment of treated versus untreated control cultures. Values represent mean of 3 independent assays. Asterisk indicates statistically significant difference ($p < 0.05$).

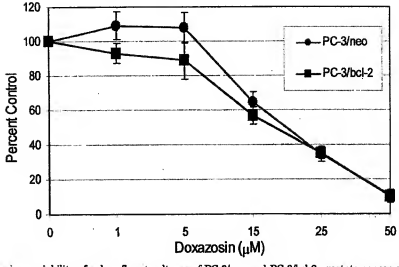

FIG. 5. Effect of doxazosin on viability of subconfluent cultures of PC-3/neo and PC-3/bcl-2 prostate cancer cells exposed to increasing concentrations of doxazosin (0.1 to 50 μM.). Cell death was determined by trypan blue exclusion assay. Values represent mean percent cell viability from 3 independent experiments.

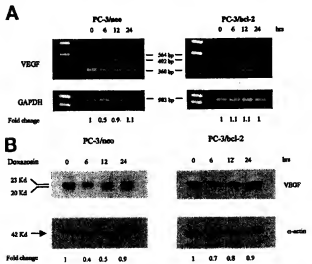

FIG. 6. Profile of mRNA expression for VEGF in doxazosin treated prostate cancer cells (*A*). RNA was extracted from PC-3/neo and PC-3/bcl-3 expressing cells after treatment with 25 μM. doxazosin for 6, 12 and 24 hours. RT-PCR was performed. Individual bands correspond to VEGF mRNA splice variant species. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. Western blot analysis shows VEGF protein expression in PC-3/neo and PC-3/bcl-2 prostate cancer cell lines (*B*). Cell lysates were prepared after treatment with 25 μM. doxazosin for 6, 12 and 24 hours. Cell lysates (30 μg.) were fractionated through 15% (weight per volume) SDS-polyacrylamide gel electrophoresis and subjected to Western blotting. Expression of α-actin served as normalizing control to confirm equivalent protein loading and transfer.

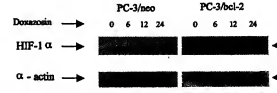

FIG. 7. Effect of doxazosin on hypoxia inducible factor-1α (*HIF-1α*) protein expression in PC-3/neo control and PC-3/bcl-2 human prostate cancer cells treated with 25 μM. doxazosin for 6, 12 and 24 hours. Nuclear protein extracts (30 μg.) from treated and untreated cells were analyzed through 7.5% (weight per volume) SDS-polyacrylamide gel electrophoresis and subjected to Western blotting.

2-fold decrease in VEGF mRNA expression in PC-3/neo cells as early as hour 6 of treatment with doxazosin (fig. 6, *A*). This down-regulation of VEGF mRNA expression was paralleled by a significant decrease in VEGF protein expression levels after doxazosin treatment (fig. 6, *B*). These changes followed a transient pattern (6 to 12 hours), characteristic of early involvement before the cellular manifestation of anoikis with VEGF mRNA and protein expression returning to baseline after 24 hours of treatment with doxazosin (fig. 6, *B*).

Since VEGF expression is regulated by hypoxia, we subsequently examined the effect of bcl-2 on hypoxia inducible factor-1α expression. Expression of hypoxia inducible factor-1α was examined in PC-3 and PC-3/bcl-2 cells after exposure to doxazosin. Figure 7 shows that doxazosin did not result in any significant changes in hypoxia inducible factor-1α protein levels in PC-3 or PC-3/bcl-2 cells.

The effect of the 3 α1-adrenoceptor antagonists in prostate cell invasion was evaluated by performing an invasion assay in a 2-chamber environment. Figure 8 shows that doxazosin and terazosin (25 μM.) inhibited prostate cancer cell invasion through Matrigel, while no effect was observed with tamsulosin. A comparable inhibition of cell invasion, as for PC-3/neo controls, was obtained for the PC-3/bcl-2 cells by doxazosin and terazosin (approximately 10% and approximately 9%, respectively) (fig. 8). Migration assays were also performed for a 24-hour treatment period in 2-chamber model with a filter membrane. These revealed similar results for PC-3/neo and PC-3/bcl-2 cells (data not shown).

DISCUSSION

Recent studies of the elucidation of anoikis mechanisms support a connection between integrin signaling and apoptosis regulatory proteins.[9,10] Indeed, integrin has been shown to support the survival of anchorage dependent cells by up-regulating bcl-2 expression.[10] In the current study the induction of prostate cancer cell death by pharmacologically relevant doses of doxazosin was preceded by a significant loss of cell attachment. To our knowledge this finding represents the first evidence of the ability of quinazoline based α1-

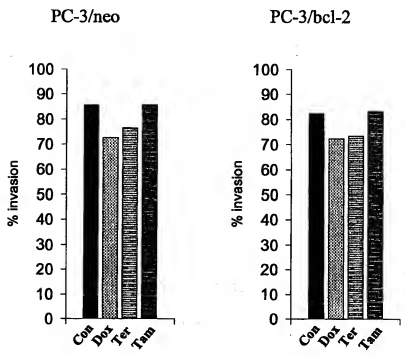

FIG. 8. Effect of α1-adrenoceptor antagonists on prostate cancer cell invasion in parental prostate cancer cells. PC-3/neo and PC-3/bcl-2 cells were labeled with fluorescent dye after invading Matrigel and passing membrane in 24-well invasion system with 8 μ. pore size in absence or presence of 25 μM. doxazosin (*Dox*), terazosin (*Ter*) or tamsulosin (*Tam*). Cells were visualized under epifluorescence microscopy. Bars indicate mean percent relative to fluorescent dye uptake by cells invaded through Matrigel and pores to lower chamber. *Con*, control.

adrenoceptor antagonists but not tamsulosin to induce anoikis and inhibit cell attachment of human prostate cancer cells.

Our findings indicate that bcl-2 over expression in prostate cancer cells exerts an antagonistic effect against this quinazoline mediated apoptotic effect by suppressing cell attachment to gelatin matrix without affecting cell invasion. Growth factors, such as transforming growth factor, basic fibroblast growth factor and VEGF, contribute to the angiogenic response of tumors via the modulation of integrin expression.[8, 12] The apparent lack of evidence for an anoikis effect on prostate cancer cell adhesion on fibronectin matrix challenges the functional involvement of integrins in the doxazosin induced inhibition of tumor epithelial and endothelial cell adhesion. As challenging as this concept may appear, it derives direct support from recent evidence suggesting that doxazosin reduced the expression of vimentin and focal adhesion kinases in PC-3 cells but had no effect on integrin linked kinase.[20] Considering the association of bcl-2 over expression with the emergence of androgen independent disease[4, 18] and its functional involvement in prostate cancer angiogenesis[20] it may be of clinical significance to dissect further its role in regulating quinazoline suppression of prostate tumor cell anoikis/angiogenesis.

VEGF is an important effector of signaling angiogenesis in the prostate under androgenic regulation. The current findings demonstrate that the quinazoline based compounds induce transient down-regulation of VEGF expression, an effect that is partially reversed by bcl-2. This decrease in VEGF expression characteristically precedes the loss of cell attachment and manifestation of apoptosis by doxazosin, pointing to potential targeting of VEGF by the quinazolines. Equally characteristic of such a response is the suppression of VEGF by bcl-2, supporting the involvement of the latter in the execution of anoikis by the quinazolines. This finding gains important mechanistic significance in view of recent evidence suggesting a role of bcl-2 as a promoter of prostate tumor angiogenesis independent of its anti-apoptotic ability.[21] The apparent lack of changes in hypoxia inducible factor-1α expression in quinazoline treated PC-3/neo and PC-3/bcl-2 prostate cells implies that a mechanism involving neither hypoxia nor its crusader hypoxia inducible factor-1α is responsible for the effect of bcl-2 on VEGF expression. Ongoing experiments are focused on determining the ability of VEGF to antagonize directly the anoikis effect of the quinazolines against endothelial cells.

In view of the current findings one could argue that if prostate tumor epithelial cells were prevented from attaching to gelatin/collagen by quinazoline derived antagonists, they activate their suicidal program of apoptosis via anoikis before an actual cytotoxic or antiproliferative effect by the drug. Direct support for this quinazoline inhibitory effect against prostate cell adhesion stems from our recent clinical data indicating that terazosin reduces prostate tumor vascularity via an anti-angiogenic effect.[17]

CONCLUSIONS

This study provides an initial mechanistic insight into the ability of quinazolines to regulate prostate growth by interfering with cell adhesion, an action potentially independent of α1-adrenoceptors. The current findings may have significant clinical implications for targeting angiogenesis by quinazoline based compounds for prostate cancer therapy. Doxazosin and terazosin may be regarded as potential anti-tumor agents for prostate cancer via the induction of anoikis and ultimately the suppression of prostate tumor cell angiogenic potential.

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